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Hyperplastic islets observed in "reversed" NOD mice treated without hematopoietic cells

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ABSTRACT

At the onset of type 1 diabetes, most of the insulin-producing pancreatic beta cells are destroyed by effector cells, and therefore, the following two factors, at a minimum, are necessary for "reversing" hyperglycemia in autoimmune diabetes; depletion of effector cells and enhancement of beta cell regeneration. In this study, we tried a novel approach for "reversing" autoimmune diabetes in a murine model. Here we show that remission could be achieved with a combination therapy of a single injection of complete Freund's adjuvant (CFA) and a single intraperitoneal injection of a pancreatic beta cell line, MIN6N-9a, in recent-onset diabetic NOD (non-obese diabetic) mice. Five out of seven mice (71%) receiving MIN6N-9a and CFA became normoglycemic within 120 days after treatment, whereas only two of nine (22%) receiving vehicle instead of MIN6N-9a achieved remission. Histological examination of pancreatic specimens from "reversed" mice showed decreased islet number, but each islet was markedly hyperplastic; being about six times larger than those from controls. Although it has been reported that hematopoietic cells such as splenocytes differentiate into insulin-producing cells and play a key role, our data indicate that they are not an absolute requirement for the "reversal" of autoimmune diabetes.

1. Introduction

Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of pancreatic beta cells responsible for insulin production. Therefore, to cure T1DM, two factors must be overcome; the autoimmune response to pancreatic beta cells must be eliminated, followed by restoration of pancreatic beta cells, most of which are considered to be lost at the disease onset. In 2001, Faustman et al. [1] reported that diabetes in non-obese diabetic (NOD) mice, a widely used murine model of T1DM, can be "reversed" to normoglycemia by combining single injection of complete Freund's adjuvant (CFA), multiple injections of semi-allogeneic splenocytes, and temporary transplantation of syngeneic islets. According to this report, CFA was thought to eliminate anti-islet autoimmunity, and the injected splenocytes to differentiate into insulin-producing cells [2]. However, there is controversy as to whether these hematopoietic cells are actually necessary to induce remission in NOD mice [3–5]. Therefore, in an effort to "cure" T1DM, we treated diabetic NOD mice using combination therapy with a single injection of CFA and a single intraperitoneal injection of a murine pancreatic beta cell line, MIN6N-9a, to temporarily control hyperglycemia; i.e. we did not include hematopoietic cells. MIN6N-9a is a cell line derived from a transgenic mouse expressing the SV40 T antigen, and has the genetic background of the NOD mouse [6,7]. Herein we report that this combination treatment could induce remission of autoimmune diabetes in NOD mice, and interestingly,

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although histological examination of pancreatic specimens from ''reversed'' mice showed a marked decrease in the number of islets, surviving islets were extremely hyperplastic being approximately six times larger than those from an age-matched normal strain of mouse.

2. Materials and methods

2.1. Mice

Female NOD/Shi and ICR mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Both strains were kept under specific pathogen-free conditions. All animal manipulations were carried out in accordance with the institutional animal care guidelines of Keio University School of Medicine, and were approved by our local animal experimentation ethics committee. Urinary glucose analysis using Tes-tape (Shionogi, Osaka, Japan) was performed twice a week, and when glycosuria was detected, the blood glucose level was determined using Glutest-Ace (Sanwa Kagaku Co., Nagoya, Japan). Mice were considered to be diabetic when blood glucose levels were above 250 mg/dl in two consecutive measurements taken 48 h apart.

2.2. Treatment of mice

Female NOD mice confirmed to be diabetic were injected with 5.0×10^6 MIN6N-9a cells intraperitoneally. MIN6N-9a cell lines were established from an insulinoma developed in one of the IT6 transgenic mice backcrossed nine times to NOD mice [6]. IT6 transgenic mice were produced by microinjection of the insulin promotor–SV40 T antigen hybrid gene into fertilized eggs from C57BL/6 mice [7]. Simultaneously, CFA (Sigma–Aldrich, St. Louis, MO) conjugated with an equal volume of saline (50 μl) was injected into each foot pad. As a control, some of the mice were injected with phosphate-buffered saline (PBS) instead of MIN6N-9a cells. MIN6N-9a cells were cultured in medium as described previously [6], washed in PBS, and teased into single cells before injection. After treatment, blood glucose levels were followed over time, and two consecutive non-fasting measurements below 200 mg/dl (at around 4:00 p.m.) were considered to represent a ''reversed'' state.

2.3. Histological analysis

The pancreas was removed from each mouse, fixed in 10% formaldehyde, and embedded in paraffin. Thin sections, 100 μm apart, were cut for staining with hematoxylin–eosin (HE). For insulin and glucagon staining, tissues were deparaffinized and stained with either anti-insulin antibody (Oriental Yeast Co., Tokyo, Japan) or anti-glucagon antibody (Nichirei Corporation, Tokyo, Japan).

2.4. Islet count and measurement of islet size

Numbers of islets in three non-serial pancreatic sections from a successfully ''reversed'' mouse were counted. Pancreatic specimens from age- and sex-matched ICR mice were used as a control. Islet size was measured in HE-stained sections and calculated using ImageJ software (National Institute of Health, Bethesda, MD). Number of islets and islet size of the ''reversed'' mice and controls were compared using the Bonferroni/Dunn test.

2.5. RT-PCR and semi-quantitative real-time PCR

NOD mice treated with MIN6N-9a cells and CFA, as described above, were killed 1 h, 1 day, or 1 week after the treatment. Total RNA was extracted from MIN6N-9a cells, the pancreas, pancreatic lymph nodes and peritoneal exudates using a RNeasy Mini kit (Qiagen, Valencia, CA). The extracted RNA was reverse transcribed using a First-Strand cDNA synthesis kit (GE Healthcare Bio-Sciences, Piscataway, NJ). The sequences used were as follows: SV40 T antigen: 5′-TGAGGCTACTGCTGACTCTCAACA-3′ and 5′-GCATGACTCAAAAAACTTAGCAATTCTG-3′, insulin: 5′-GACCTTCAGACCTTGGC-3′ and 5′-GCAGTAGTTCTCCAGCTGG-3′, GAPDH: 5′-TGGTGAAGGTCGGTGTGAAC-3′ and 5′-CCATGTAGTTGAGGTCAATGAAGG-3′. Semi-quantitative RT-PCR was conducted for IFN-γ, TNF-α, IL-4, IL-10, TGF-β1, Foxp3, and GAPDH (internal control) in an ABI Prism 7500 Fast sequence detector (Applied Biosystems, Foster City, CA) using assay-on-demand real-time PCR kits. cDNA obtained from pancreatic lymph nodes from both successfully ''reversed'' and not ''reversed'' mice were used. The obtained mRNA level was expressed relative to that of the GAPDH PCR product amplified from the same sample ((sample PCR product/GAPDH PCR product) × constant).

3. Results

3.1. ''Reversal'' of diabetes in treated diabetic NOD mice

Female diabetic NOD mice (18–40 week-old) were treated with CFA and either MIN6N-9a or PBS at the onset of diabetes. Five out of seven mice had glucose levels in the normoglycemic range by 120 days after treatment with CFA and MIN6N-9a. On the other hand, only two of nine receiving CFA and PBS achieved normoglycemia (Fig. 1A and B).

3.2. Histological characteristics of islets from ''reversed'' mice

In mice in which normoglycemia was restored, histological examination of the pancreas revealed some unique characteristics. First, the number of islets was markedly decreased (Fig. 2A). Second, the surviving islets in the MIN6N-9a and CFA treated ''reversed'' mice were all hyperplastic, and the degree of insulitis in these islets was consistently ''peri-insulitis stage'' (Fig. 2B). The hyperplastic islets had an abundance of insulin-positive cells, thought to be beta cells (Fig. 2C), and few glucagon-positive cells were detected (Fig. 2D). Also, a small number of contracted islets were identifiable with HE staining in successfully ''reversed'' mice. However, most of the cells in these contracted islets were glucagon positive, insulin negative, and devoid of leukocyte infiltration. The size of the islets from ''reversed'' mice (12 islets from three mice) was $(6.2 \pm 1.0) \times 10^{-2}$ mm^2 (mean ± S.E.), whereas those in NOD mice before treatment, i.e. in recent-onset diabetic female